Novel Selective Estrogen Receptor Modulator (SERM) OSU-ERβ-12 Demonstrates Anti-fibrotic Efficacy

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation

with *honors research distinction* in the undergraduate colleges of The Ohio State University.

by

Abigail Klein

The Ohio State University

December 2020

Project Advisor: Dr. Christopher Coss, Department of Pharmaceutics and Pharmacology

Acknowledgements

Thank you to Dr. Christopher Coss for the opportunity to participate in research within his lab. Thank you to Hanna Radomska, for generating the cellular data on which these experiments were based, Samuel Kulp for his mentorship and help with the uterotrophic assay, and Timothy Helms for providing the CCl_4 efficacy data and mentorship during this project.

Support generously provided by the Drug Development Institute, Comprehensive Cancer Center, and Division of Pharmaceutics and Pharmacology as well as the CREATES Fellowship.

Abstract

Purpose: Based on demonstrated ERβ-selective transcriptional activity in a cellular model, it was hypothesized that *in vivo,* efficacious doses of the novel ERβ agonist OSU-ERβ-12 could be determined that have minimal activity in ERα-dependent tissues. These doses were hypothesized to have an anti-fibrotic effect in a Non-Alcoholic Steatohepatitis (NASH) liver disease model.

Design: We used uterotrophic stimulation in an estrogen-naïve female mouse model to assess ERα activity. Briefly, groups of mice were administered doses of the ERβ selective agonist OSU-ERβ-12 and compared to a control ERβ-selective ligand, LY500307. The ERα effects of each ligand were characterized using bodyweight-normalized uterine tissue weights. Then doses of OSU-ERβ-12 were given to mice treated with 6 weeks of CCl_4 to establish efficacy of OSU-ERβ-12 as an antifibrotic compound. Liver sections were stained with picrosirius red and the staining was then quantified using computer analysis.

Results: OSU-ERβ-12 demonstrated ERβ-selective activity at doses <30 mg/kg, with 10 mg/kg being the highest selective dose. This dose was also efficacious at reducing the amount of fibrotic tissue picrosirius red staining seen in a liver section that was challenged with CCl_4. LY500307 showed increased toxicity and decreased efficacy as compared to OSU-ERβ-12.

Conclusions: Consistent with cellular activity data, uterotrophic stimulation data and the CCl_4 challenge data suggest that 10 mg/kg doses of OSU-ERβ-12 are both ERβ-selective and efficacious in a CCl_4 model of liver disease.

Introduction

Liver disease is a cause of ever-increasing concern to the world, as it is a largely unmet crisis in terms of healthcare. Liver disease that causes fibrosis in the form of Non-Alcoholic Fatty Liver Disease (NAFLD) or Non-Alcoholic SteatoHepatitis (NASH) predisposes patients to liver cancer, especially Hepatocellular Carcinoma (HCC). It is thought that NAFLD and NASH will soon overtake viral hepatitis as the leading cause of liver cancer worldwide. Because there is currently no approved therapy for NAFLD or NASH, any breakthroughs in this area have the potential to save or improve millions of lives (Bataller, 2005).

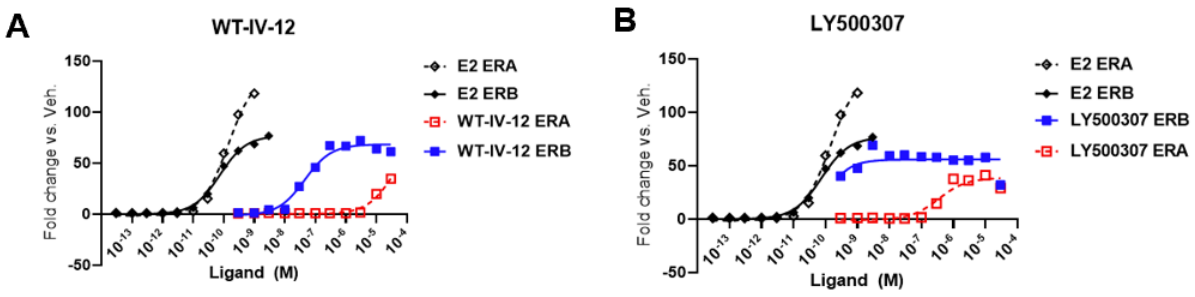
Liver disease is characterized by accumulation of fibrotic tissue in the liver, including collagen and other extracellular matrix proteins (Bataller, 2005). Fibrosis has many negative outcomes, including cirrhosis, liver failure, and liver cancers. Fibrosis in the liver is mostly thought to be permanent, and there is currently no way to treat NASH or associated liver failure outside of liver transplant. There are several therapeutics that aim to help reverse liver fibrosis or prevent fibrosis from advancing, spurred by recent studies in which some reversibility of liver fibrosis was seen in mice. There are currently no approved therapies for human patients with NASH, and so this is an urgent need in healthcare.

One of the vital premises for a study into potential estrogen therapeutics for the treatment of NASH is the presence of sexual dimorphism in liver cancer and liver fibrosis. Males have significantly higher rates of liver cancer than females, both in mice and humans, which indicates that liver cancer is somewhat reliant on sex hormone levels in the body. It is thought that estrogens may play some role in helping prevent or treat liver cancer and its precedent, sexually dimorphic conditions (Wu, 2019). The estrogen receptor is expressed in many different tissues within the body, including the uterus, ovaries, prostate, brain, breast, and bones. The estrogen receptor has two predominant forms; estrogen receptor α (ERα) and estrogen receptor β (ERβ) that are expressed in different amounts throughout the body. ERα is expressed in high amount in the uterus, breast and ovaries, as well as prostate and brain, while ERβ is expressed in the colon, prostate, ovary, bone marrow and brain. ERβ seems to be a good clinical target because of its implication in inflammation, cancer, and cardiovascular disease (Mohler, 2010).

Exogenous therapeutic estrogens that target estrogen-receptor α are well documented in the study of cancer, but have potentially lethal side effects and cause castration in males. It is therefore not desirable to treat cancers with non-selective estrogens like the most predominant endogenous estrogen, estradiol. However, it is theorized that ERβ can be selectively targeted to treat liver disease without the toxic effects from ERα. It is hoped that the antifibrotic effects seen in response to estrogens can be achieved by selectively modulating ERβ without stimulating ERα, bypassing the toxic side effects caused by ERα (Mohler, 2010).

A medication of this nature would be considered a selective estrogen receptor modulator (SERM), and there is currently a compound that was tested as a potential ERβ selective SERM developed by Eli Lilly labelled LY500307. LY500307 is the most clinically advanced ERβ agonist, and is the class standard that will be analyzed in this paper. This molecule was analyzed in Phase I and II clinical trials but was ultimately abandoned due to lack of efficacy in prostatic disease (Clinical Trial# NCT01097707). The second medication that will be analyzed is a novel carborane compound developed at The Ohio State University by W. Tjarks, and it is one of the many carborane based estrogens that Tjarks developed and tested for potential selectivity and efficacy *in vitro*. The prospective compound that will be analyzed in this paper is the OSU-ERβ-12, formerly known as WT-IV-12.

The estrogen receptor selectivity for OSU-ERβ-12 and LY500307 were established using ERE-LUC (estrogen response element luciferase reporter assay) driven transactivation assay. In this assay, ER-null HEK293 cells were transiently transfected with either human ERα or ERβ, and the ability of increasing concentrations of each compound to stimulate expression of a luciferase transgene were assessed (Kim and Coss, 2013). **Figure 1A** shows the activation of the estrogen receptors in response to drug concentrations of the novel selective estrogen-receptor modulator (SERM) OSU-ERβ-12 and Estradiol (17-βE_2, E2).



C

nM	ERA EC_{50}	ERB EC_{50}	ERA/ERB
E2	0.14	0.075	2.0
WT-IV-12 (new)	>30,000	44.9	>668
LY500307	346	<0.3	>1153

Figure 1. ERE-LUC driven transactivation assay in HEK293 cells transiently transfected with either human ERα or ERβ. **(A)**OSU-ERβ-12 (WT) demonstrates ERβ selectivity, but reduced potency compared to 17-βE2. **(B)** LY500307 demonstrates ERβ selectivity with similar potency to 17-βE2. **(C)** Tabulation of EC_{50} values for WT-IV-12 and LY500307 reveal ERB selectivity of ~500- and 1000-fold, respectively. (Data generated by Hanna Radomska)

The estradiol control shows no initial separation in the stimulation of ERβ and ERα as is expected. However, OSU-ERβ-12 shows a distinct difference in the onset of receptor activity. The blue line represents the activity shown by ERβ, and the onset of transcriptional activity occurs at much lower concentrations of compound than ERα (>668 fold). These data support an opportunity for the development of ERβ selective dosing of this compound such that ERβ but not ERα are activated thus avoiding toxic effects of ERα. There is a similar trend for the class standard, the LY500307 compound made by Eli Lilly (**Figure 1B**, >1153-fold). The EC_{50} and the selectivity of the two novel compounds is shown in the table (**Figure 1C**), showing that both compounds have a significant window of selectivity, greater than ~500 fold each for ERβ. However, potency (EC_{50}) of OSU-ERβ-12 for ERβ was reduced compared to LY500307, suggesting larger doses of OSU-ERβ-12, as compared to LY500307, are required for efficacy.

Based on previous work showing the potential for ERβ-modulation to impact fibrotic liver disease (Zhang, 2013) (Ponnusamy, 2017) (Zhang, 2018),we hypothesized ERβ-selective doses of the novel carborane SERM OSU-ERb-12 could demonstrate anti-fibrotic efficacy absent ERα-mediated side effects. The following report demonstrates the process of establishing ERβ-selective dose levels and then testing their tolerability and anti-fibrotic efficacy in the CCl_4 model of fibrosis.

Methods

Establishing an Estrogen Receptor β Selective Dose with Uterotrophic Assay

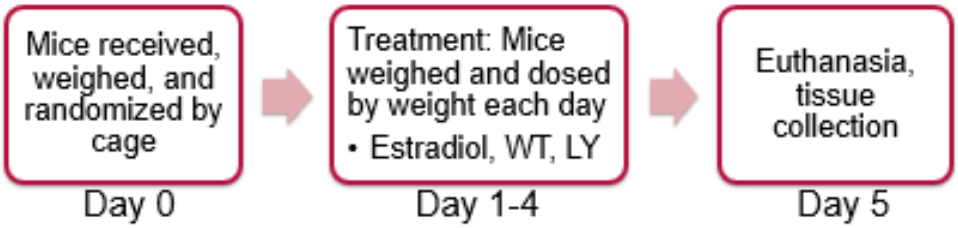


Figure 2. Diagram showing the treatment schedule for the uterotrophic assay. Mice were received, weighed, randomized, and ear tagged on Day 0, weighed and dosed with treatment compounds on Day 1-4, and sacrificed on Day 5.

65 total C57/Bl6 female mice who were 20 days of age on day 0 were ear tagged and weighed in preparation for dosing the next day. Mice were randomly separated into treatment groups of 5 mice per group. Groups consisted of 5 different doses of the novel selective estrogen receptor modulator (SERM) OSU-ERβ-12, 4 different doses of the current class standard SERM LY500307 produced by Eli Lilly, an estradiol group, and three respective vehicle control groups. Doses of the OSU-ERβ-12 compound were 3, 10, 30, 100 and 300 mg/kg in a vehicle of 5% DMSO, 5% Tween 80 in water. Doses of the LY500307 compound were 0.03, 0.1, 0.3, and 1.0 mg/kg in 1% MC, 0.25% Tween 80 in water. The two SERM compounds were dosed orally through gavage, along with the two corresponding vehicle controls. The estradiol group was dosed subcutaneously in corn oil as a 50 μg/kg dose with a subcutaneous corn oil vehicle control.

Each mouse was weighed and dosed daily for 4 days. On the fifth day the mice were sacrificed, and tissue was collected. The uterus was extracted without puncturing, weighed, and then punctured and blotted to get both a wet weight with fluids inside the uterus and a blotted weight with the fluids removed. Cross sections of the uterine left horn were saved in formalin and imaged using 200x magnification.

OSU-ERβ-12 Efficacy in CCl_4 NASH Model

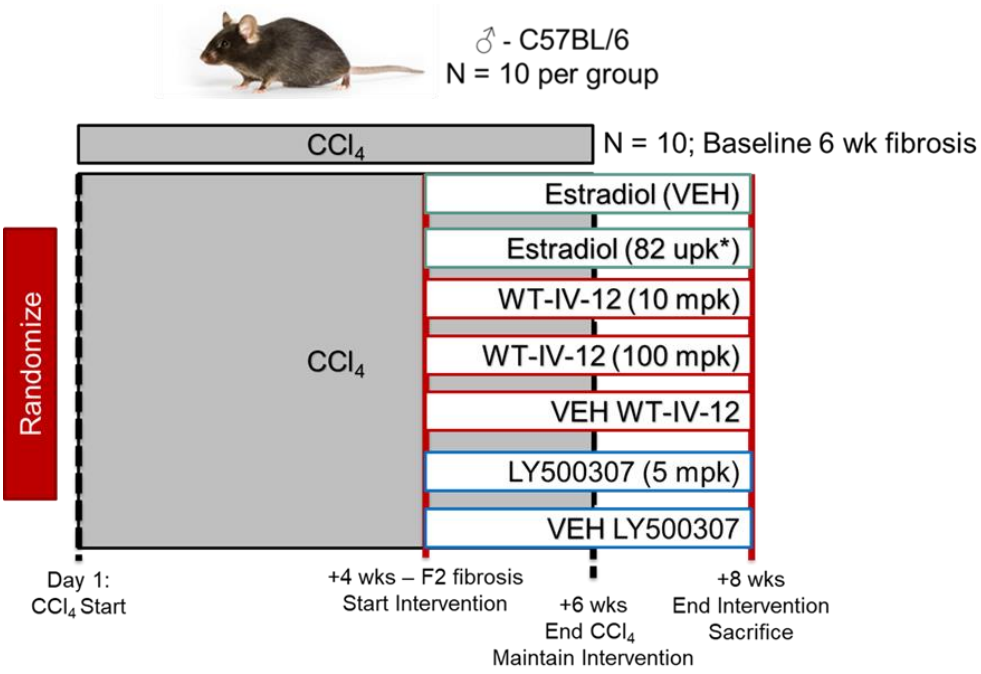


Figure 3. Diagram showing treatment plans for each mouse group. One group dosed with only CCl_4 for six weeks, and all other mouse groups treated with CCl_4 for six weeks with intervention treatment starting on the fourth week. Seven intervention treatment groups; Sub-cutaneous (SC) estradiol and SC vehicle, PO OSU-ERβ-12 (WT) compound in doses of 10 mg/kg and 100 mg/kg as well as the vehicle for this compound, and a PO LY500307 at 5 mg/kg and the corresponding vehicle.

80 total C57/Bl6 male mice were randomly assigned to treatment groups with 10 mice per treatment group. All groups were treated with escalating doses of 50:50 carbon tetrachloride (CCl_4) for six weeks through oral gavage (Kim, 2017). The stage of fibrosis was determined by the length of CCl_4 administration as shown by a previous pilot study. (Helms) Based on this pilot work, at 4 weeks of CCl_4 administration mice were expected to have the equivalent of human F2 stage liver fibrosis as confirmed by histological analyses. Treatment with intervention compounds was started in the 4th week, with seven different treatment groups (**Figure 2**). One group was not treated to help characterize potential spontaneous fibrosis resolution. Treatment groups included an estradiol group of 82 μg/kg in 5% DMSO in corn oil, 10 and 100 mg/kg treatment groups of the novel SERM OSU-ERβ-12 in 5% DMSO, 5% Tween80 in water, and a 5 mg/kg group of the class standard SERM LY500307. There were also three vehicle groups, one

for each of the treatments. The estradiol group and 5% DMSO and corn oil vehicle were both dosed subcutaneously, while all other treatment groups were dosed orally through gavage. Estradiol was included here as a positive control as there is established efficacy in preventing NAFLD and NASH in other CCl_4 models (Liu, 2004) (Besse-Patin, 2017). CCl_4 treatment continued for another 2 weeks and was then stopped. The group not treated with an intervention was then sacrificed to characterize spontaneous fibrosis resolution and baseline fibrosis. The primary objective was to quantitatively assess fibrosis through picrosirius red (PSR) staining of collagen deposits. Picrosirius red staining is a way to visualize collagen in liver fibrosis, and can be quantified through computer imagining (Standish, 2006). A secondary objective of this experiment was to determine if there were toxic ERα effects present in male mice by looking at the urogenital tract weights to determine if there was atrophy as compared to the vehicle control.

Results

Experimental Rationale

The goal of these experiments was to establish the efficacy of the novel SERM OSU-ERβ-12 and to establish a dose that was shown to be selective for estrogen receptor β. A selective dose first needed to be established to determine the doses that would be tested in the liver fibrosis efficacy model.

The uterotrophic assay was chosen to help establish a selective dose of OSU-ERβ-12 because uterine weight of estrogen naïve mice is a potent indicator of estrogen receptor α activity (Mohler, 2010). A small amount of ERα activity will cause measurable effects on an estrogen naïve uterus, such that a dose of a compound that does not cause an increase in uterine weight should be ERβ selective. We hypothesized that the highest dose group absent ERα stimulation is the highest ERβ selective dose.

A CCl_4 liver fibrosis model was used to demonstrate extensive fibrosis similar to the fibrosis seen in NASH, and to test the ability of multiple therapeutic estrogens to treat ongoing fibrosis in the context of an existing fibrotic burden. Fibrosis is an important indicator of disease because it is strongly related to patient outcomes and often dictates treatment (Hagström, 2017). In the CCL4 model, fibrotic pathology is distinct from those as seen in metabolic disorder caused by obesity, but as a well-established model it has notable benefits and drawbacks (Oligschlaeger, 2020).

Urogenital tract atrophy and biomarkers for hepatic cholestasis were also measured in the male mice from the CCl_4 efficacy study to look at potential toxic ERα effects in male mice.

ERβ Selective Dosing of OSU-ERβ-12

The uterotrophic assay is intended to determine a dose that is free from toxic ERα effects in C57Bl/6 estrogen naïve female mice. This assay shows that at doses < 30 mg/kg there is no uterine stimulation in OSU-ERβ-12 (WT) as determined by blotted uterine weight relative to body weight (**Figure 4A**). There was no uterine stimulation for any of the LY doses administered. (**Figure 4B**)

A histological analysis of the OSU-ERβ-12 (WT) compound at a low and high dose levels compared both to the OSU-ERβ-12 (WT) vehicle and estradiol shows lack of stratification of endometrial cells in the vehicle control and 3 mg/kg dose of OSU-ERβ-12 (WT). The vehicle and 3 mg/kg groups show normal juvenile uterine histomorphology: single layer of columnar endometrial epithelium, and a densely populated endometrial stroma. **(Figure 5A and 5C)** The high 100 mg/kg dose of OSU-ERβ-12 (WT) shows histomorphological features consistent with exposure to estrogens: hyperplastic, stratified endometrial epithelium, and hypertrophied, eosinophilic endometrial stroma.

Establishing Efficacy of OSU-ERβ-12 in a CCl_4 Liver Fibrosis Model

After six weeks of oral CCl_4 dosing, the first group of mice (only treated with CCl_4 and no intervention) were sacrificed to help show a baseline of fibrosis at the end of the CCl_4 challenge. When compared to vehicle treated mice sacrificed 2 weeks later, these data also show how much fibrosis spontaneously resolves following cessation of CCl_4 administration. (**Figure 6A**) The three vehicle control groups were compared to the CCl_4 group with the assumption that the vehicle control does not help reduce fibrosis, and so any resolution of fibrosis after the cessation of CCl_4 administration was spontaneous and not treatment related. The CCl_4 group shows a significant difference in the amount of fibrosis seen through PSR staining of histological liver section (**Figure 6B**) as compared to all three vehicle control groups. Vehicle control groups were also treated for six weeks with CCl_4, but vehicle administration began on week four and continued for two weeks after cessation of CCl_4 administration (**Figure 2**).

After treatments was concluded on week eight, all mouse groups were sacrificed and PSR staining in treatment groups were compared to the vehicle controls. All treatment groups were compared to their respective vehicle controls. Subcutaneous estradiol did not reduce PSR staining when compared to its vehicle control (**Figure 7A**), with the histological representation of this in **Figure 7B**.

There was a significant reduction of PSR staining in the LY500307 liver slides (**Figure 8A**), with a p value of 0.0404 following a Tukey's multiple comparisons test analysis. The histological staining visually shows this reduction as well (**Figure 8B**).

The OSU-ERβ-12 compound (Abbreviated here as WT), shows a marked decrease in PSR staining. (**Figure 9A**) This trend is seen for both doses, showing that a lower dose of the OSU-ERβ-12 compound is still efficacious at reducing PSR staining in a CCl_4 fibrosis model (**Figure 9B**).

Alkaline Phosphatase (ALP) is a marker for hepatic cholestasis, because an increase in serum ALP shows decreased or stopped bile flow from the liver (Alvaro, 2006). Estrogens have been shown to induce hepatic cholestasis, which is a toxic effect caused by damage or blockage to the biliary ducts of the liver (Alvaro, 2006). Estradiol and 5 mg/kg LY500307 both show a significant increase in ALP, but OSU-ERβ-12 only shows a significant increase in ALP at the

high dose of 100 mg/kg. The low, efficacious, 10 mg/kg dose of OSU-ERβ-12 does not show a significant increase in serum ALP levels.

Urogenital tract weight atrophy (standardized to body weight) is an indicator of ERα toxicity in hormone sensitive male secondary sex organs. This is thought to be due to ERα-mediated suppression of androgen synthesis through the hypogonadal-pituitary-gonadal (HPG) axis (Lindzey, 1998). In this study, the UGT tract included the seminal vesicle, coagulating gland, prostate and urinary bladder. Samples were standardized to individual body weights and compared to their corresponding vehicle controls. There is a significant reduction in UGT weight for all groups from their respective vehicle for all groups except for the 10 mg/kg OSU-ERβ-12 (WT) group. Tukey's multiple comparisons test E2 and LY groups differed from their respective controls ($p<0.0001$) and OSU-ERβ-12 (WT) differed from the OSU-ERβ-12 (WT) vehicle ($p=0.0054$) (**Figure 11A**). The 10 mg/kg OSU-ERβ-12 (WT) group was then compared to both the LY and E2 groups directly, and there is significantly less UGT weight reduction in the OSU-ERβ-12 (WT) 10 mg/kg group as compared to these groups.

There was no significant reduction in testes weight for the LY500307 compound or OSU-ERβ-12 (WT) compound at either dose, although both the LY dose and 100 mg/kg OSU-ERβ-12 (WT) dose showed a downward weight trend. The estradiol showed significant testes weight reduction as expected.

Conclusions

This report has shown that ERβ agonism is a viable therapeutic approach to address liver fibrosis like that seen in NASH. Both LY500307 and OSU-ERβ-12 showed efficacy at doses shown to be ERβ selective, but this effect was not seen in the estradiol treated mice, indicating that ERβ modulation alone is producing this antifibrotic effect, and not ERα or a non-selective therapeutic estrogen. Our data indicate that it is possible to selectively target ERβ to achieve estrogen related effects absent the side effects that come from unwanted ERα such as thromboembolic events or UGT atrophy in males.

Though the high 100 mg/kg dose of OSU-ERβ-12 showed some efficacy in the CCl_4 liver fibrosis model, it showed increased toxicity and was deemed to be an unselective dose because of the estrogenic activity seen in the uterotrophic assay. However, selective doses of OSU-ERβ-12 are readily achieved in mice, with the highest selective dose being 10 mg/kg. This dose was also deemed to be efficacious in the CCl_4 liver fibrosis model.

This compound has incredible potential in therapeutics to be one of the first treatments for NASH, both as a preventative and a treatment that can help reduce current fibrosis levels. This compound shows improved selectivity and toxicity as compared to the previous class standard, LY500307. The 10 mg/ kg selective dose of OSU-ERβ-12 shows significantly less UGT deterioration and cholestasis in male mice as compared to LY500307. Though LY500307 was shown to be efficacious at a dose of 5 mg/kg, there were also significant toxicities that are not readily ascribable to endocrine toxicity. At 10 mg/kg, the OSU-ERβ-12 compound was efficacious, but without some of the negative side effects measured, such as cholestasis or UGT deterioration. There were increased side effects with the high dose of OSU-ERβ-12, indicating that the side effects are only seen with a non-selective dose of the compound.

In conclusion, OSU-ERβ-12 fits nicely into the landscape of NASH therapeutics as a novel estrogen therapeutic as endocrine therapies have not been explored in NASH (Brodosi, 2016). However, OSU-ERβ-12 is not the first SERM to be utilized in the treatment of disease. In fact, SERM drugs such as tamoxifen and raloxifene have long been used in the treatment of cancers and they are relatively well tolerated in both men and women (Wibowo, 2016). The potential for a SERM treatment that targets ERβ is well studied in multiple diseases, including

prostate and chronic inflammatory diseases, but not severe liver disease. Because the pathophysiology of NASH is complex and there are many different aspects involved, targeting NASH from different angles is desirable, and there is great promise in targeting estrogens based on the degree of sexual dimorphism seen in NASH patients (Kur, 2020).

In addition, the experimental model that we used to depict NASH fibrosis was purposeful in starting treatment after establishing liver damage with the CCl_4. This model is closer to what would be seen in a patient, as most patients do not start intervention drugs before they have significant fibrosis. Most NASH patients will have a significant amount of fibrosis in their liver before starting treatment, so a drug that can ameliorate existing fibrosis as well as prevent new fibrosis is critical. Our data suggests that OSU-ERβ-12 can ameliorate fibrosis, not only slowing it from the start of the CCl_4 challenge, but also ameliorating existing fibrosis and slowing the formation of more fibrotic tissue. This study also shows that OSU-ERβ-12 shows considerable improvement as compared to LY500307, both in the sense of toxicity as well as efficacy in a mouse model. OSU-ERβ-12 is promising, not only in terms of SERMs, but also as a treatment for NASH and other disease states that are mediated by ERβ.

Figures

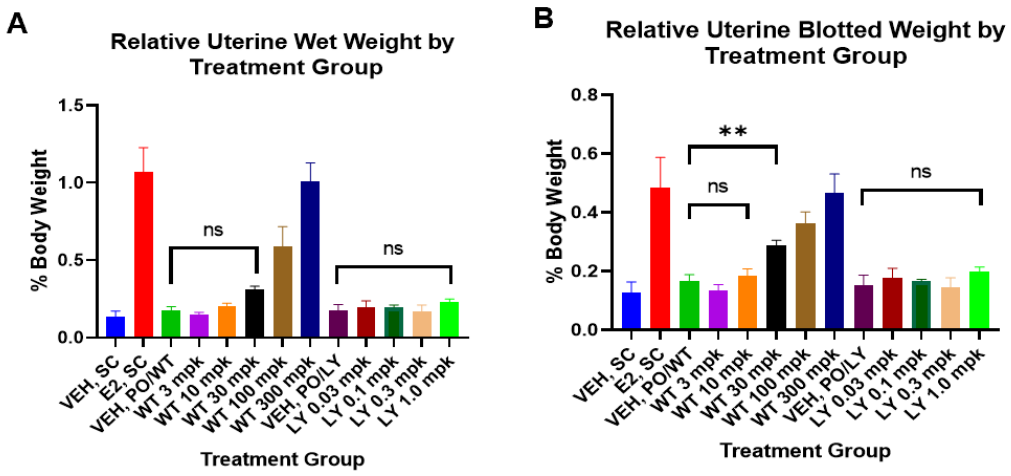


Figure 4. **Uterine weight by treatment group (A)** Relative uterine wet weight by treatment group. OSU-ERb-12 (WT) doses of 100 and 300 mg/kg significantly increased weights compared to controls ($p<0.0001$), Dunnett's multiple comparisons test. The LY500307 treatment groups were not different from the vehicle controls. **(B)** Relative uterine blotted weight by treatment group. WT-IV-12 doses of 30, 100 and 300 mg/kg significantly increased weights compared to controls ($p<0.0001$), Dunnett's multiple comparisons test. The LY500307 treatment groups were not different from the vehicle controls.

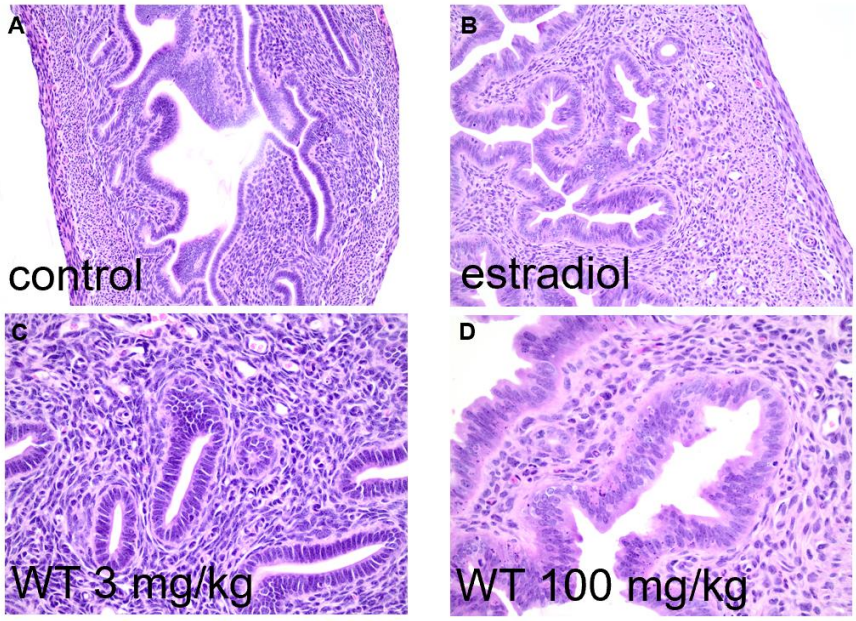


Figure 5. **Representative photomicrographs of uterine left horn from experimental mice. (A)** 200x total magnification of the left uterine horn of a mouse from the 5% DMSO, 5% Tween 20 vehicle (OSU-ERβ-12 control) group. Shows normal juvenile uterine histomorphology: single layer of columnar endometrial epithelium, and a densely populated endometrial stroma. **(B)** 200x magnification of the left uterine horn of a mouse from the subcutaneous estradiol group. Shows histomorphological features consistent with exposure to estrogens: hyperplastic, stratified endometrial epithelium, and hypertrophied, eosinophilic endometrial stroma. **(C)** 400x magnification of the uterine left horn of a mouse from the 3 mg/kg OSU-ERβ-12(WT) group. Shows comparatively normal juvenile uterine histomorphology. **(D)** 400x magnification of the left uterine horn of a mouse from the 100 mg/kg OSU-ERβ-12 group. Shows histomorphological features consistent with changes in Estrogen group.

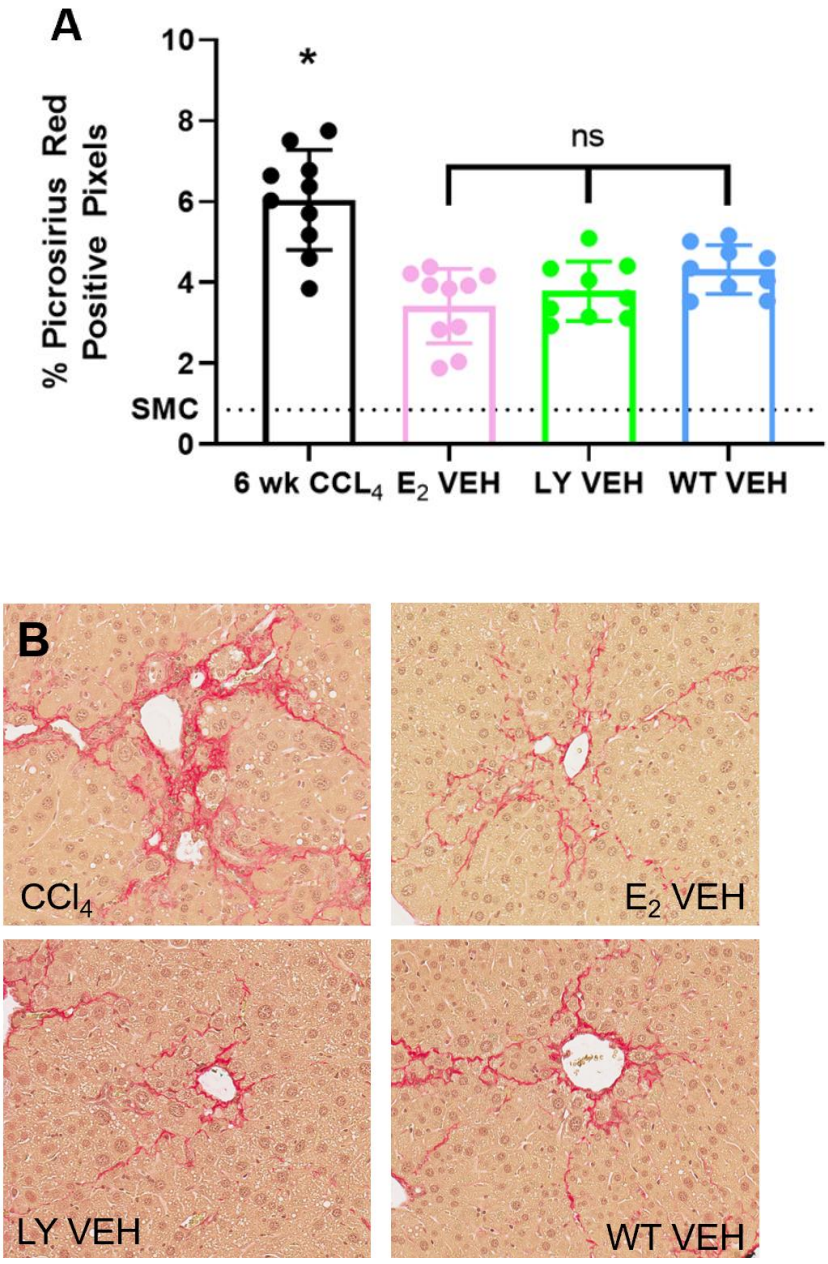


Figure 6. **(A)** Percent of positive picrosirius red pixels shown on each liver slide. Figure comparing the six-week CCl_4 group to the vehicle intervention groups (sacrificed at 8 weeks and given 4 weeks of vehicle intervention treatment). There was no significance between the three vehicle controls, but the amount of fibrosis was significantly greater in the six week CCl_4 group compared to all three vehicle groups by Tukey's multiple comparisons analysis ($p<0.0001$). **(B)** Histological representation of groups stained with Picrosirius Red.

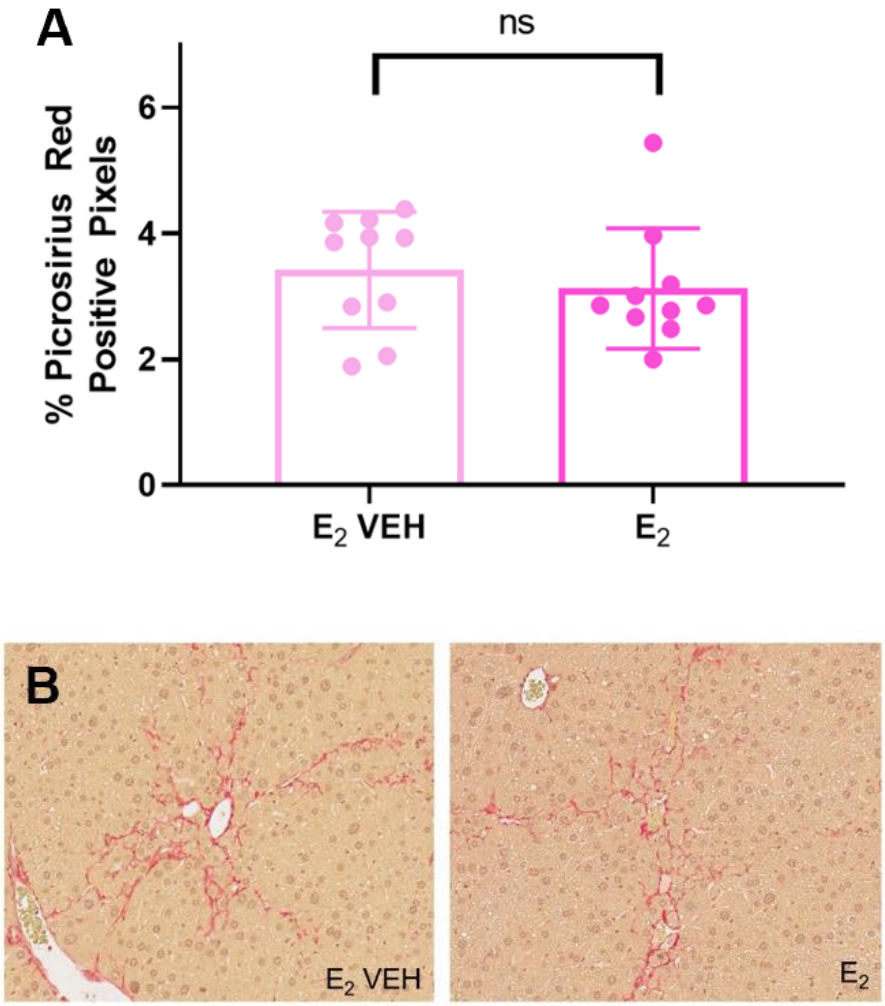


Figure 7. **(A)** Percent of positive picrosirius red pixels (PSR) shown on the SQ estradiol slide compared to the SQ estradiol vehicle slide. There was no significant difference between the vehicle control group and the estradiol intervention group, indicating no reduction of fibrosis. Tukey's multiple comparisons analysis ($p=0.4905$). **(B)** Representative histological staining with Picrosirius Red for the estradiol group and respective vehicle control.

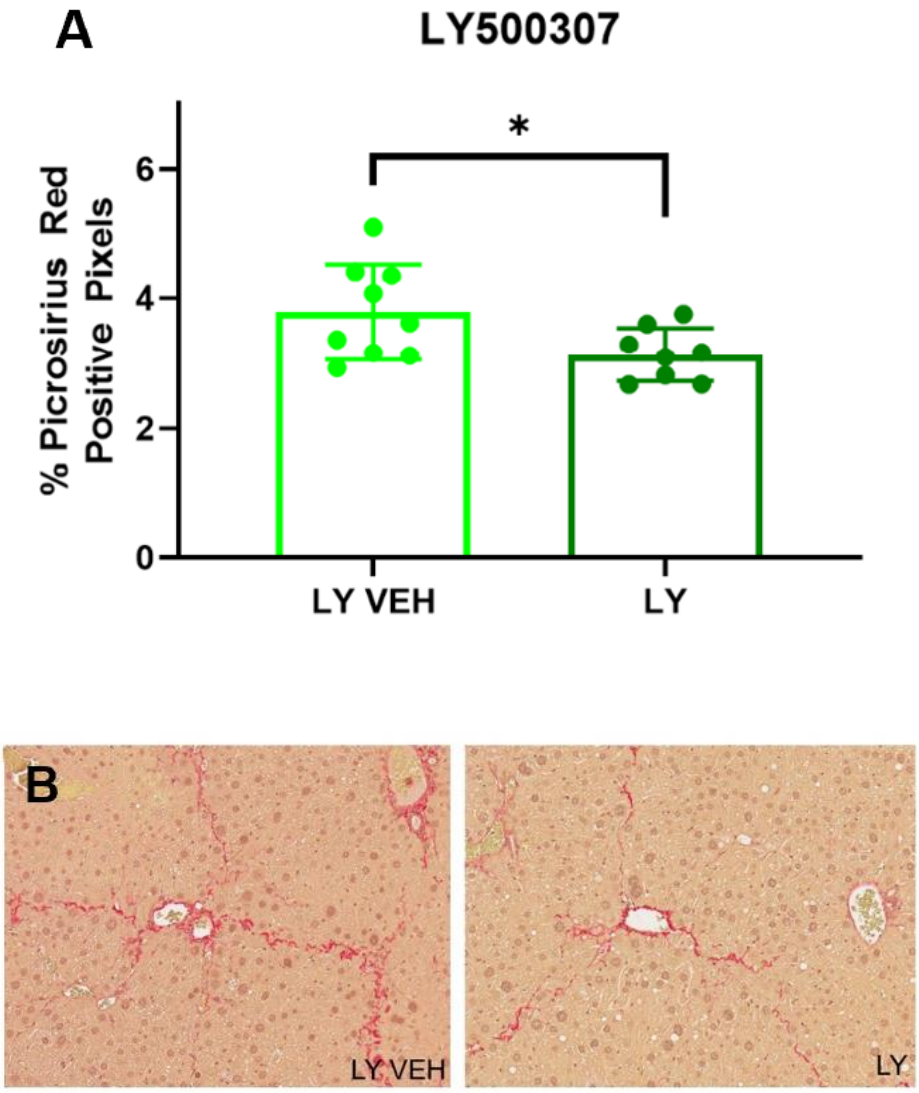


Figure 8. **(A)** Percent of positive picrosirius red pixels shown on the PO LY500307 liver slide compared to the PO LY500307 vehicle slide. There was a significant reduction of fibrosis after LY administration as compared to the vehicle control. Tukey's multiple comparisons analysis (p=0.0404). **(B)** Representative histological staining with Picrosirius Red for the LY group and respective vehicle control.

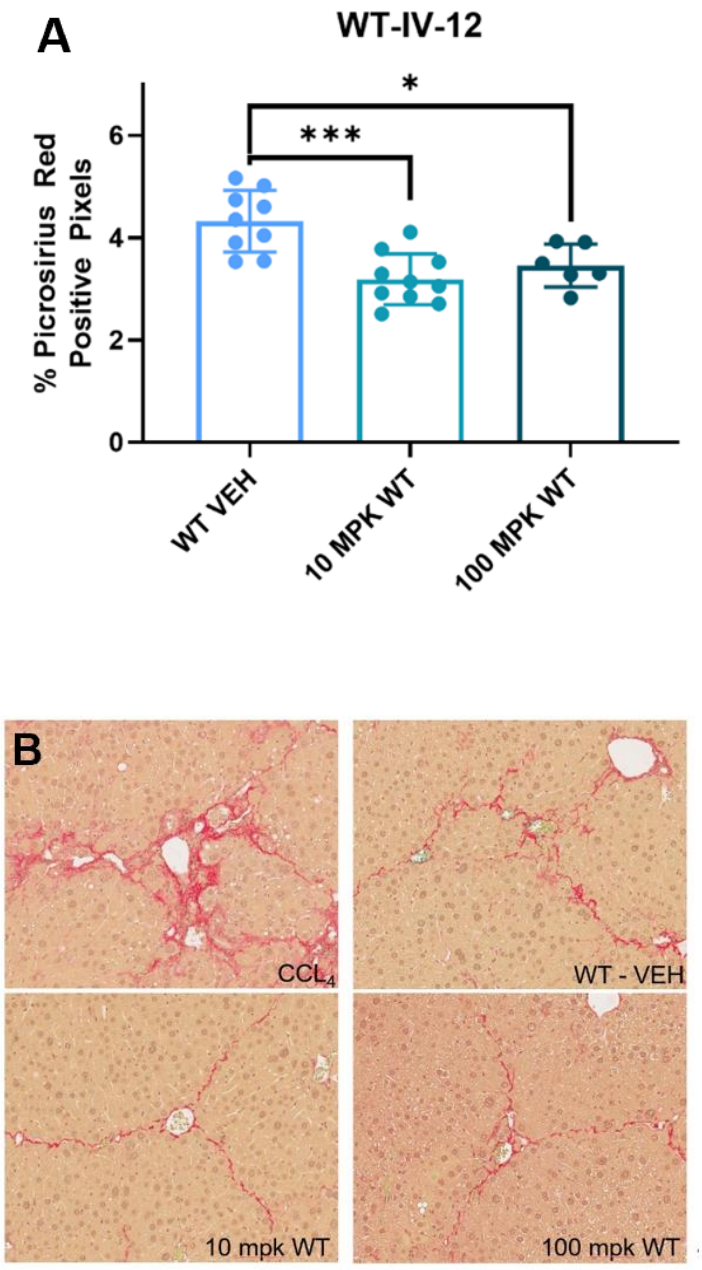


Figure 9. **(A)** Percent of positive picrosirius red pixels shown on the PO OSU-ERβ-12 (WT) liver slides for 10 mg/kg and 100 mg/kg doses compared to the PO OSU-ERβ-12 (WT) vehicle slide and six week CCl_4 only slide. There was a significant reduction of fibrosis after OSU-ERβ-12 (WT) administration as compared to the vehicle control for both dose levels. Tukey's multiple comparisons analysis WT vehicle vs 10 mg/kg WT (p=0.0003) and WT vehicle vs 100 mg/kg WT (p=0.0127). **(B)** Representative histological staining with Picrosirius Red for the OSU-ERβ-12 (WT) dose groups, respective vehicle control, and six week CCl_4 group.

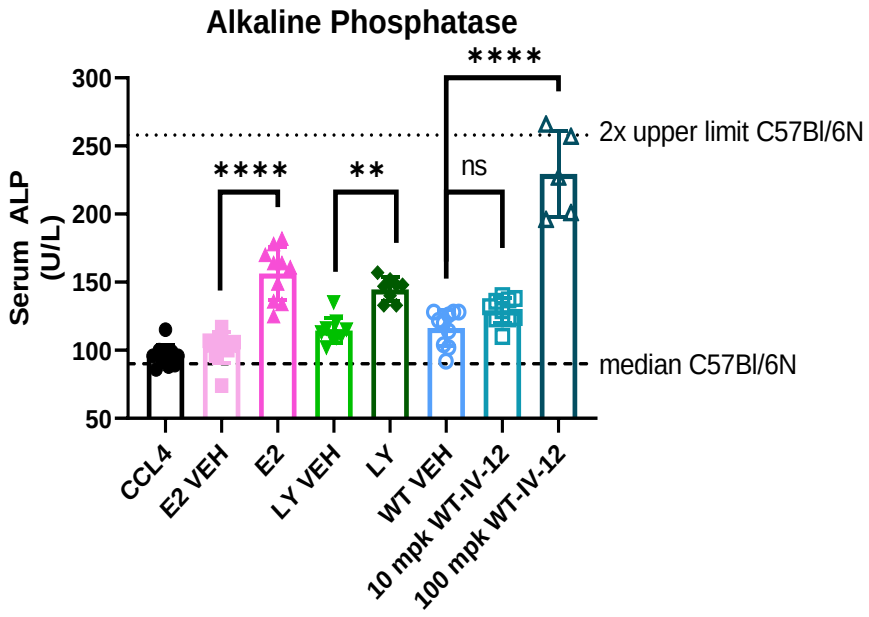


Figure 10. **(A)** Serum ALP (U/L) in each treatment group with horizontal reference lines representing the median and 2x upper limit serum ALP levels in a C57Bl/6N mouse. There was a significant increase in serum ALP compared to the respective vehicle for estradiol, LY and the 100 mg/kg dose of OSU-ERβ-12 (WT) administration. There was no significant difference from the vehicle for the 10 mg/kg dose of OSU-ERβ-12 (WT). Tukey's multiple comparisons analysis WT vehicle vs 10 mg/kg WT ($p=0.4686$). E2 vehicle vs E2, OSU-ERβ-12 (WT) 10 mg/kg vs 100 mg/kg and WT vehicle vs 100 mg/kg WT ($p<0.001$). LY vehicle vs LY ($p=0.0010$).

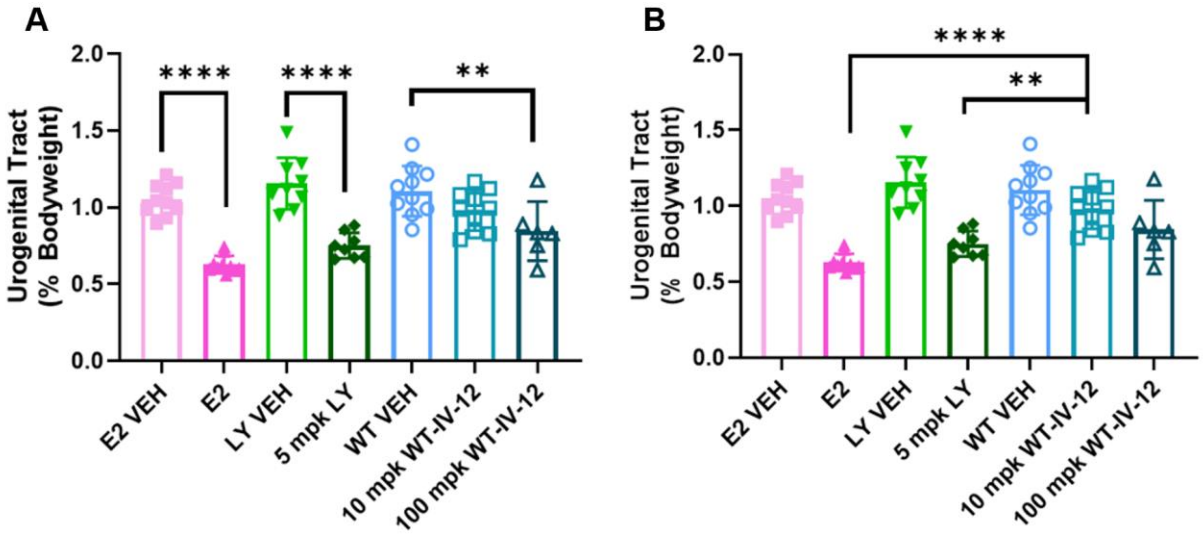


Figure 11. **(A)** UGT tract weights standardized to body weight for each treatment group compared to their respective controls. There is a significant reduction for all group from their respective vehicle for all groups except for the 10 mg/kg OSU-ERβ-12 (WT) group. Tukey's multiple comparisons test E2 and LY groups differed from their respective controls ($p<0.0001$) and OSU-ERβ-12 (WT) 100 mg/kg differed from the OSU-ERβ-12 (WT) vehicle ($p=0.0054$). **(B)** UGT tract weights for the 10 mg/kg OSU-ERβ-12 (WT) group are compared to weights of the LY and E2 groups. Tukey's multiple comparisons test E2 vs OSU-ERβ-12 (WT) ($p<0.0001$) and LY vs OSU-ERβ-12 (WT) 100 mg/kg ($p=0.0095$).

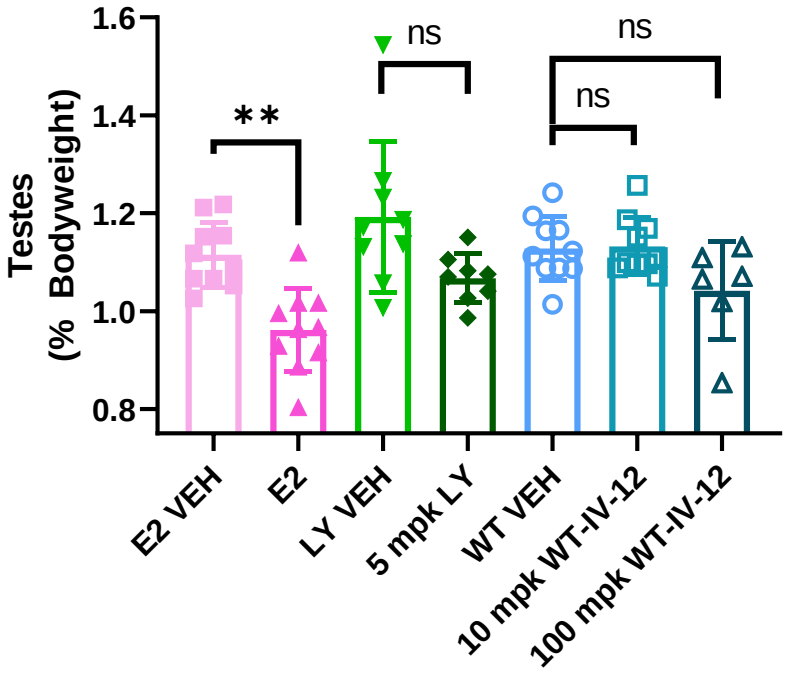


Figure 12. **(A)** Testes weights standardized to body weight for each treatment group compared to their respective controls. There is a significant reduction for the estradiol group compared to the estradiol vehicle. Tukey's multiple comparisons test E2 vs vehicle (p=0.0043). No other group showed a significant difference from its respective control group.

Citations

Alvaro, D., Mancino, M. G., Onori, P., Franchitto, A., Alpini, G., Francis, H., Glaser, S., & Gaudio, E. (2006). Estrogens and the pathophysiology of the biliary tree. *World J Gastroenterol, 12*(22), 3537-3545. https://doi.org/10.3748/wjg.v12.i22.3537

Bataller, R., & Brenner, D. A. (2005). Liver fibrosis. *J Clin Invest, 115*(2), 209-218. https://doi.org/10.1172/JCI24282

Besse-Patin, A., Léveillé, M., Oropeza, D., Nguyen, B. N., Prat, A., & Estall, J. L. (2017). Estrogen Signals Through Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α to Reduce Oxidative Damage Associated With Diet-Induced Fatty Liver Disease. *Gastroenterology, 152*(1), 243-256. https://doi.org/10.1053/j.gastro.2016.09.017

Brodosi, L., Marchignoli, F., Petroni, M. L., & Marchesini, G. (2016). NASH: A glance at the landscape of pharmacological treatment. *Ann Hepatol, 15*(5), 673-681. https://doi.org/10.5604/16652681.1212318

Hagström, H., Nasr, P., Ekstedt, M., Hammar, U., Stål, P., Hultcrantz, R., & Kechagias, S. (2017). Fibrosis stage but not NASH predicts mortality and time to development of severe liver disease in biopsy-proven NAFLD. *J Hepatol, 67*(6), 1265-1273. https://doi.org/10.1016/j.jhep.2017.07.027

Kim, Y. O., Popov, Y., & Schuppan, D. (2017). Optimized Mouse Models for Liver Fibrosis. *Methods Mol Biol, 1559*, 279-296. https://doi.org/10.1007/978-1-4939-6786-5_19

Kur, P., Kolasa-Wołosiuk, A., Misiakiewicz-Has, K., & Wiszniewska, B. (2020). Sex Hormone-Dependent Physiology and Diseases of Liver. *Int J Environ Res Public Health, 17*(8). https://doi.org/10.3390/ijerph17082620

Lindzey, J., Wetsel, W. C., Couse, J. F., Stoker, T., Cooper, R., & Korach, K. S. (1998). Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. *Endocrinology, 139*(10), 4092-4101. https://doi.org/10.1210/endo.139.10.6253

Liu, Q. H., Li, D. G., Huang, X., Zong, C. H., Xu, Q. F., & Lu, H. M. (2004). Suppressive effects of 17beta-estradiol on hepatic fibrosis in CCl4-induced rat model. *World J Gastroenterol, 10*(9), 1315-1320. https://doi.org/10.3748/wjg.v10.i9.1315

Mohler, M. L., Narayanan, R., Coss, C. C., Hu, K., He, Y., Wu, Z., Hong, S. S., Hwang, D. J., Miller, D. D., & Dalton, J. T. (2010). Estrogen receptor beta selective nonsteroidal estrogens: seeking clinical indications. *Expert Opin Ther Pat, 20*(4), 507-534. https://doi.org/10.1517/13543771003657164

Oligschlaeger, Y., & Shiri-Sverdlov, R. (2020). NAFLD Preclinical Models: More than a Handful, Less of a Concern? *Biomedicines, 8*(2). https://doi.org/10.3390/biomedicines8020028

Ponnusamy, S., Tran, Q. T., Thiyagarajan, T., Miller, D. D., Bridges, D., & Narayanan, R. (2017). An estrogen receptor β-selective agonist inhibits non-alcoholic steatohepatitis in preclinical models by regulating bile acid and xenobiotic receptors. *Exp Biol Med (Maywood), 242*(6), 606-616. https://doi.org/10.1177/1535370216688569

Standish, R. A., Cholongitas, E., Dhillon, A., Burroughs, A. K., & Dhillon, A. P. (2006). An appraisal of the histopathological assessment of liver fibrosis. *Gut, 55*(4), 569-578. https://doi.org/10.1136/gut.2005.084475

Warner, M., Huang, B., & Gustafsson, J. A. (2017). Estrogen Receptor β as a Pharmaceutical

Target. *Trends Pharmacol Sci*, *38*(1), 92-99. https://doi.org/10.1016/j.tips.2016.10.006
Wibowo, E., Pollock, P. A., Hollis, N., & Wassersug, R. J. (2016). Tamoxifen in men: a review of adverse events. *Andrology*, *4*(5), 776-788. https://doi.org/10.1111/andr.12197
Wu, Y., Yao, N., Feng, Y., Tian, Z., Yang, Y., & Zhao, Y. (2019). Identification and characterization of sexual dimorphism-linked gene expression profile in hepatocellular carcinoma. *Oncol Rep*, *42*(3), 937-952. https://doi.org/10.3892/or.2019.7217
Zhang, B., & Wu, Z. Y. (2013). Estrogen derivatives: novel therapeutic agents for liver cirrhosis and portal hypertension. *Eur J Gastroenterol Hepatol*, *25*(3), 263-270. https://doi.org/10.1097/MEG.0b013e32835ab5dc
Zhang, B., Zhang, C. G., Ji, L. H., Zhao, G., & Wu, Z. Y. (2018). Estrogen receptor β selective agonist ameliorates liver cirrhosis in rats by inhibiting the activation and proliferation of hepatic stellate cells. *J Gastroenterol Hepatol*, *33*(3), 747-755. https://doi.org/10.1111/jgh.13976